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Amplified In Situ Hybridization With Peptide Nucleic Acid Probes for Differentiation of *Mycobacterium tuberculosis* Complex and Nontuberculous *Mycobacterium* Species on Formalin-Fixed, Paraffin-Embedded Archival Biopsy and Autopsy Samples

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Key Words: In situ hybridization; ISH; Tuberculosis; *Mycobacterium tuberculosis; Mycobacterium;* Peptide nucleic acid probe; PNA probe

Abstract

The aim of this study was to evaluate sensitivity and specificity of in situ hybridization (ISH) using peptide nucleic acid (PNA) probes and tyramide-based amplification for the differentiation between Mycobacterium tuberculosis *(MTB) and mycobacteria other than tuberculosis (MOTT) on formalin-fixed, paraffin-embedded tissue samples. We performed ISH simultaneously with both probes on 86 specimens from different organs: 70 obtained at autopsy and 16 by biopsy, all with a histologic evidence of mycobacterial infection confirmed by Ziehl-Neelsen–positive staining. Taking culture as the "gold standard," the sensitivity and the specificity of the MTB probe were 100% (41/41) and 95% (38/40), respectively. In only 2 cases ISH failed to identify mycobacteria. Culture results were not available in 3 cases. We propose ISH as a relatively simple and rapid method to differentiate mycobacteria on formalin-fixed, paraffin-embedded specimens (it is more specific than usual histologic stains) and as an alternative to polymerase chain reaction, allowing the morphologic evaluation of positive bacilli.*

Mycobacterial infections are a worldwide emergency. Tuberculosis, caused by infection with species of the *Mycobacterium tuberculosis* complex (MTB), is still the most important cause of death from an infectious agent. During the last 2 decades, tuberculosis prevalence in the industrialized countries has increased both from the HIV infection epidemic and immigration from developing countries.[1,2] Infections from *Mycobacterium* species other than *M tuberculosis* (MOTT) are commonly observed opportunistic diseases in people with AIDS.

Pathologists frequently are asked for the diagnosis of mycobacterial infections on formalin-fixed, paraffin-embedded tissue samples. The evaluation of H&E-stained slides can give useful information from the morphologic features of the lesions, which are usually different in MTB and MOTT infections. Morphologic criteria, however, may be less informative in the setting of immunodeficiency, eg, in people with AIDS in whom the classic granulomatous reaction of MTB infection cannot be adequately mounted or when the presence of mycobacteria is associated with minimal tissue damage. The demonstration of mycobacteria on formalin-fixed, paraffin-embedded specimens can be difficult. The most widely used histochemical stain, Ziehl-Neelsen, is simple and fast, but it has a low sensitivity further reduced by formalin fixation and does not allow for differentiation between MTB and MOTT, which is relevant for planning the treatment. Alternative methods are based mainly on amplification of genomic sequences from extracted DNA or RNA. Polymerase chain reaction (PCR), amplifying several different tracts of the mycobacteria genome, has been used successfully.[3-10] The PCR method is relatively fast, taking from several hours to a few days according to the length of

Table 1
Organs on Which In Situ Hybridization Was Performed

Organ	No. of Samples
Autopsy cases (n = 70)	
Lymph node	35
Spleen	12
Lung	11
Liver	4
Brain	3
Small bowel	3
Large bowel	1
Pancreas	1
Biopsy cases (n = 16)	
Lymph node	8
Bone marrow	6
Skin	1
Small bowel	1

Table 2
Culture Results

Species	No. of Samples	
	Autopsy Cases (n = 70)	Biopsy Cases (n = 16)
Mycobacterium tuberculosis	34	7
Mycobacterium avium	34	5
Mycobacterium intracellulare	1	0
Mycobacterium scrofulaceum	1	0
Mycobacterium chelonae	0	1
Not performed	0	3

the extraction method, and far more sensitive than the Ziehl-Neelsen staining method, enabling differentiation between MTB and MOTT species. However, the method requires special equipment, its sensitivity can be affected by fixation, and, above all, it is accepted that specificity can be reduced by false-positive cases due to contamination during one of the steps of the procedure.[11,12] Furthermore, as PCR can amplify a very low number of copies of mycobacterial genomic sequences, true-positive PCR results can be difficult to interpret when no morphologic hallmarks of the disease are found.

In situ hybridization (ISH) also has been performed on selected clinical specimens to demonstrate and to identify mycobacteria.[13] Recently, the use of peptide nucleic acid (PNA) probes has been reported for the identification of culture-grown mycobacteria[14-16] and then applied for diagnostic purposes on sputum specimens.[17]

PNA probes are nucleic acid analogues in which the sugar phosphate backbone of DNA and RNA is substituted with a polyamide formed by repetitive units of N-(2-aminoethyl) glycine to which nucleobases are attached covalently. Hybridization can be performed after specific base pairing to complementary sequences of DNA and RNA. The hydrophobic structure of PNA probes has been claimed to give a better penetrance through the mycobacterial cell wall, thus yielding higher sensitivity.[14]

The in situ hybridization procedure is relatively simple, requires only minimal equipment, and permits morphologic evaluation of positive signals. Furthermore, the simultaneous use of MTB and MOTT PNA probes provides an internal control of reaction, thus further reducing the risk of false-positive results.

The aim of the present study was to evaluate the sensitivity and specificity of ISH with PNA probes and tyramide-based amplification for the identification of mycobacteria on formalin-fixed, paraffin-embedded tissue samples.

Materials and Methods

Study Population

A total of 86 Ziehl-Neelsen–positive samples were examined, 70 of which were obtained at autopsy and 16 by biopsy. All were routinely formalin-fixed and paraffin-embedded. The length of fixation varied from 2 days to 1 month for autopsy tissues, while biopsy specimens were fixed for a maximum of 3 days. Sources and numbers of the organs are listed in **Table 1**.

All autopsy and 15 biopsy samples were from HIV-positive patients. All autopsy samples were from patients who had positive blood culture results for mycobacteria within 1 month before death. Isolates included in the study were identified by the AccuProbe rRNA hybridization assay (GenProbe, San Diego, CA) or phenotypic classification by using standard biochemical assays.[18] The numbers and types of mycobacteria are given in **Table 2**. Blood culture results also were available for 13 patients of the biopsy group.

In Situ Hybridization

Serial 4-µm-thick sections were cut and deparaffinized in xylene, followed by 5-minute stepwise immersions in methanol alcohol in descending concentrations, ending in distilled water. To block endogenous peroxidase, 15-minute incubation in 2% H_2O_2 in 80% methanol alcohol was performed during deparaffinization.

Pretreatment was in 2 phases: 15 minutes in a solution of proteinase K (10 µg/mL) in 50 mL of tris(hydroxymethyl)aminomethane (Tris) buffer at 37°C; and two 5-minute periods in a microwave oven at 780 W in a jar containing citric acid (1 mmol/L concentration). Slides were refreshed for approximately 30 minutes, then dehydrated in ethanol alcohol and air-dried. Fifty microliters of the PNA probe,[14] diluted in hybridization buffer[14] to a final concentration of 5 nmol/L (MOTT probe) or 10 nmol/L (MTB probe), was applied to each slide, covered with a cover glass, and

then placed in a humidity chamber in an oven at 55°C and hybridized for 90 minutes. Cover glasses were removed in distilled water, and then 30 minutes of stringent washing in Tris-buffered saline containing Tween 20 (TBST) buffer in an oven at 55°C was performed. Slides were rinsed in water and TBST buffer twice for 10 minutes each and incubated with a peroxidated antibody, anti–fluorescein isothiocyanate (P5100, DAKO A/S, Glostrup, Denmark), diluted 1:1,000 in TBST buffer for 30 minutes. Subsequently, slides were rinsed in TBST buffer 3 times for 5 minutes each; incubated with biotinyl tyramide for 15 minutes; rinsed in TBST buffer 3 times for 5 minutes each; incubated with streptavidin diluted 1:50 in Tris buffer; rinsed in TBST buffer, Tris-buffered saline buffer, and Tris buffer for 3 minutes each; and then incubated with diaminobenzidine for 10 minutes. Slides were stained with hematoxylin for 30 seconds.

Statistical Analysis

Using the 2 probes simultaneously allowed us to consider for the calculation of sensitivity and specificity only cases with a positive result for one probe and a negative result for the other. A double-positive or a double-negative result is inconclusive and, therefore, was not considered for statistical analysis. The sensitivity and specificity of ISH were calculated taking culture as the "gold standard." The 95% confidence intervals were computed using the exact binomial distribution approach.

Results

In situ hybridization gave a positive result in 68 of 70 autopsy cases and in all biopsy cases. We found simultaneous positivity for MTB and MOTT in none of the cases. Taking culture results as the gold standard for identification of mycobacteria, all autopsy and biopsy cases classified as MTB infections were positive for MTB by ISH (34/34 and 7/7, respectively) **Table 3**. Among the 36 autopsy MOTT culture-positive cases, 32 were positive with the MOTT probe. Two cases had a blood culture positive for *Mycobacterium avium* 3 weeks and 1 week, respectively, before death, but a positive ISH result for MTB. The morphologic features of both cases were considered suggestive of MTB infection. Furthermore, these 2 cases were studied by performing a nested PCR on extracted DNA for an IS6110 sequence of MTB complex, for which the result was positive, and a PCR for a sequence common to MTB and MOTT, which gave a negative result.

Two results were inconclusive. Both were classified as MOTT infections based on culture results and had not been identified by ISH: one because the slides were considered not interpretable on morphologic examination owing to excessive proteolytic effect, and the other because the slides were repeatedly negative with both MTB and MOTT probes. The latter also was negative by ISH for glyceraldehyde 3-phosphate dehydrogenase messenger RNA and by PCR, thus suggesting probable loss of the RNA target.

Table 3
Sensitivity and Specificity of Peptide Nucleic Acid In Situ Hybridization Compared With Culture Results*

	Culture Result		
	MTB+	MOTT+	Total
In situ hybridization result			
MTB+/MOTT–	41	2	43
MOTT+/MTB–	0	38	38
MTB–/MOTT–	0	1	1
Not interpretable	0	1	1
Total	41	42	83

MTB, *Mycobacterium tuberculosis*; MOTT, mycobacteria other than tuberculosis.
* Sensitivity of the MTB probe and specificity of the MOTT probe: 41/41 = 100% (95% confidence interval, 93%-100%). Specificity of the MTB probe and sensitivity of the MOTT probe: 38/40 = 95% (95% confidence interval, 77%-97%).

In the 13 biopsy cases with culture identification, ISH gave concordant results (7/7 MTB cases and 6/6 MOTT cases). The 3 other cases without cultural data were classified by ISH as MTB (n = 2) and MOTT (n = 1) infections.

As expected, morphologic evaluation of ISH staining was simple in cases with a high bacterial load and even at low magnification (×20), while a prolonged high magnification scan was required in some paucibacillary cases. Related to Ziehl-Neelsen staining, ISH seemed to identify a comparable or somewhat lower number of mycobacteria in all cases but one (a lung specimen with MTB infection). In most of the cases, however, true-positive staining was morphologically distinguishable from the background of ISH reaction **Image 1** and **Image 2**.

With culture as the gold standard, the overall sensitivity of PNA ISH for MTB identification was 100% (41/41; 95% confidence interval [CI], 93%-100%) and the overall specificity (which also means the capacity of detection of MOTT cases), 95% (38/40; 95% CI, 83%-99%) (Table 3).

Discussion

We applied ISH with PNA probes and tyramide amplification for the identification of mycobacterial infections on formalin-fixed, paraffin-embedded tissues. Until now, ISH had been applied in sporadic cases of suspected mycobacterial infection.[13] We planned a retrospective evaluation of the sensitivity and specificity of PNA probes for differentiation of MTB and MOTT infections on Ziehl-Neelsen–positive autopsy and biopsy samples routinely treated for

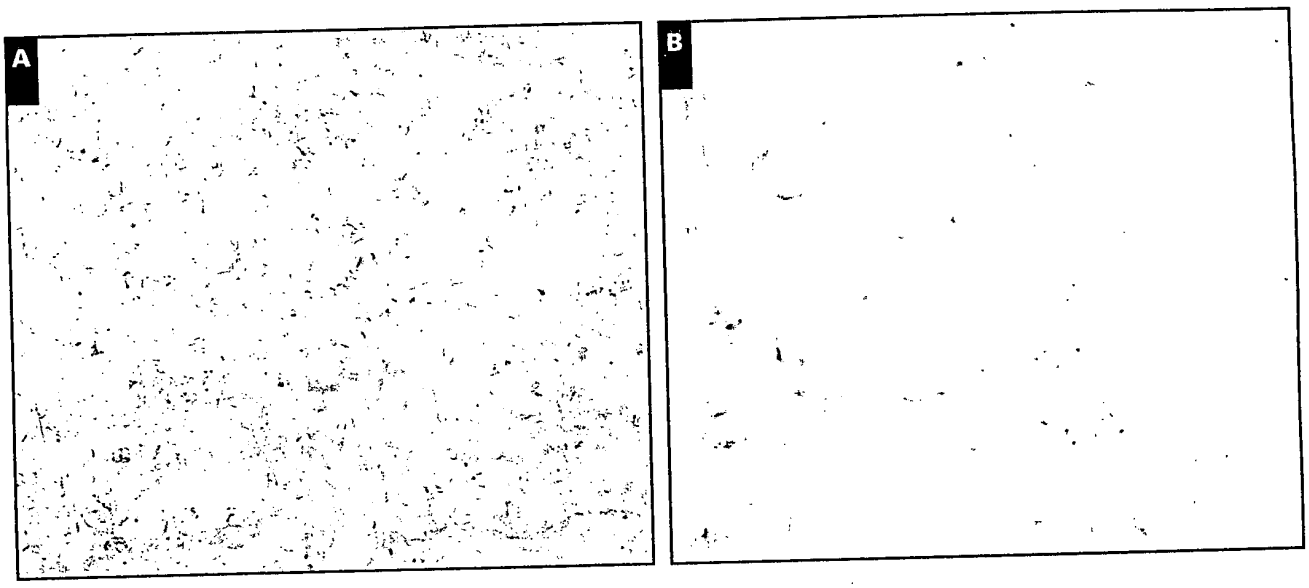

Image 1 Lung. Positive in situ hybridization for *Mycobacterium tuberculosis* (**A**, H&E, ×40; **B**, H&E, ×100).

morphologic evaluation. PNA probes are hydrophobic analogues of nucleic acid, with a repetitive aminoacidic sequence instead of the sugar backbone of DNA and RNA. Fluorescein-labeled PNA probes have been applied for the identification of cultured bacteria and on sputum, showing high sensitivity and specificity.[14-17] The simultaneous use of MTB and MOTT probes gives an internal control of the reaction by considering as true-positive results only those that were positive with one probe and negative with the other, although a double positivity actually may indicate a true double infection. The major advantage of ISH is that it allows morphologic evaluation of the positive signals, which highly contributes to avoiding false-positive results. The main drawbacks of the method are probably the failure of MOTT probes to recognize some MOTT species, which are, however, rarely found in our epidemiologic setting, as well as the fact that probes recognize RNA molecules, which can be partly or entirely destroyed during the fixation and inclusion steps. Moreover, these probes identify only the whole bacteria, ie, an active infection, which can further reduce the sensitivity of the test compared with PCR for mycobacterial DNA; however, it implies a higher specificity and a more simple interpretation of the positive results.

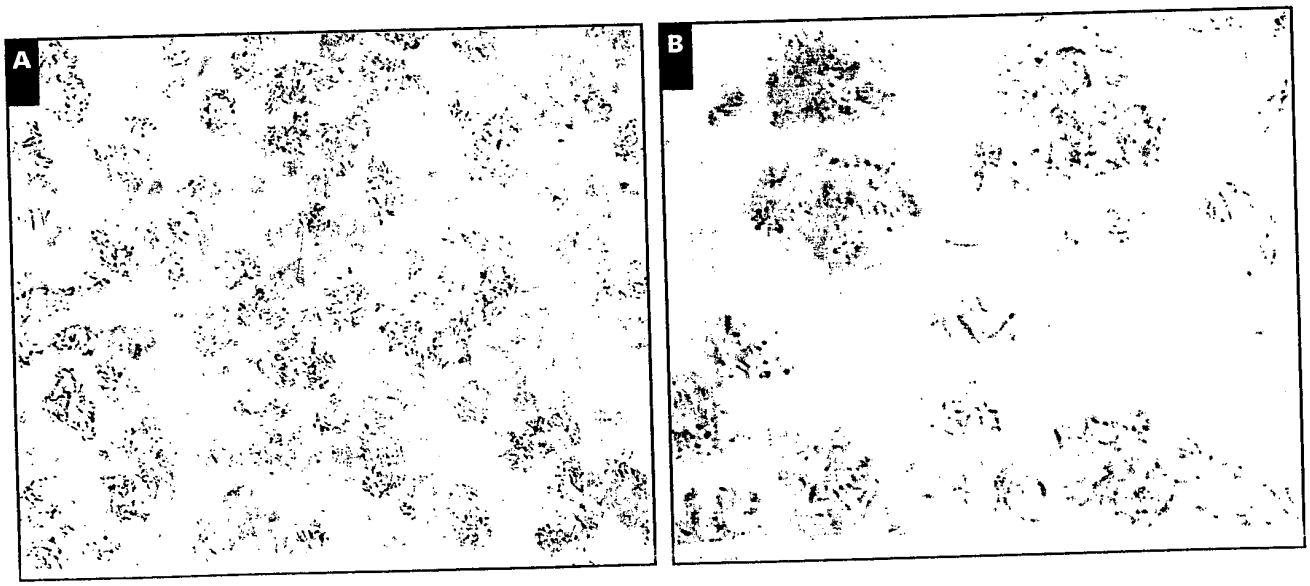

Image 2 Lymph node. Positive in situ hybridization for *Mycobacterium avium* (**A**, H&E, ×40; **B**, H&E, ×100).

The sensitivity of ISH was 100%, ie, ISH correctly identified all MTB infections. The specificity was lower (95%) as 2 cases were identified by culture as *M avium* infections but had a histopathologic pattern suggestive of MTB infection and a PCR-positive result for *M tuberculosis* with a nested PCR on extracted DNA (although a less sensitive PCR method for a sequence common to both *M tuberculosis* and *M avium* failed to amplify the microbial DNA). Accordingly, the sensitivity of ISH for MOTT had the same value, being lower than that of the MTB probe.

Our results demonstrate a very high sensitivity of the method, taking culture results as the gold standard. We had only 1 false-negative result, and another result could not be evaluated as we were unable to find pretreatment conditions allowing acceptable morphologic preservation of the section. The first case was a Ziehl-Neelsen–positive *M avium* lymph node infection, which was presumably diagnosed as a MOTT infection according to the histopathologic pattern. This false-negative result probably was due to loss of the integrity of mycobacterial ribosomal RNA, as supported by the negative result with ISH for the messenger RNA of glyceraldehyde 3-phosphate dehydrogenase. Taking these 2 cases into account, the sensitivity of the MOTT probe decreased slightly to 90% (95% CI, 77%-97%).

In none of the cases did we observe double positivity. As indicated, one of the major potential problems of ISH, ie, preservation of morphologic details after the pretreatment and posttreatment steps of the protocol, did not allow identification in only 1 case. The use of an enzymatic proteolytic treatment followed by microwave incubation offers the best compromise between sensitivity and morphologic preservation compared with either enzymatic or microwave treatment in different concentrations and at different temperatures (data not shown). It is impossible to eliminate the tyramide amplification step, as ISH alone has a very low sensitivity, and, furthermore, amplification highly reduces the need for stronger proteolytic treatment and tissue damage. In the present study, we chose to work with biotinyl-tyramide and peroxidase-labeled streptavidin. We preferred the conventional optic examination to a fluorescein detection method, because in our opinion, the optic examination gives the possibility of a better evaluation of the relationship between positive signals and tissue structures. However, we agree that this choice may be conditioned by personal preferences. Anyway, the interpretation of the reaction was possible with few or no problems in most autopsy cases, all showing a high bacterial load. By contrast, the morphologic interpretation can require prolonged scanning of the slides at high magnification in paucibacillary cases and in samples, for example, bone marrow biopsy specimens, in which the number of positive bacteria can be exceedingly low.

To our knowledge, this is the first study that demonstrates the reliability of a tyramide-amplified PNA-ISH procedure for the molecular differentiation of mycobacterial species on formalin-fixed, paraffin-embedded tissues.

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